Effects of bc_1-site electron transfer inhibitors on the absorption spectra of mitochondrial cytochromes *b*

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Changes are described that are brought about by antimycin, NoHOQnO, funiculosin, myxothiazol and mucidin in the α-, β- and γ-absorption bands of reduced and oxidized cytochromes *b* in the isolated complex bc_1 form beef heart mitochondria. The inhibitors can be divided into 2 groups. Antimycin, funiculosin and NoHOQnO are likely to shift the spectrum of *b*-562 and compete for specific binding with complex bc_1, with each other but not with myxothiazol and mucidin. The spectral effects of the latter two inhibitors are more difficult to interpret and may involve contributions not only from *b*-562 but from *b*-566 as well. The existence of 2 independent inhibitor binding-sites in the complex bc_1 corroborates the Q-cycle hypothesis.

Cytochrome b *Q cycle* *Spectral shift* *Respiratory chain* *Electron transfer inhibitor*

1. INTRODUCTION

Electron transfer in the ubiquinol : cytochrome *c* oxidoreductase segment of the mitochondrial respiratory chain is blocked specifically by a number of antibiotics such as antimycin, 2-alkyl-4-hydroxyquinoline *N*-oxide, funiculosin, mucidin, myxothiazol, as well as by many synthetic CoQ antagonists (review [1–3]). These inhibitors are specific ligands of the cytochrome bc_1 complex and can be anticipated to affect certain characteristics of the redox centres involved in the reactions inhibited. Indeed, funiculosin, NoHOQnO and myxothiazol have been shown recently to modulate midpoint redox potentials of mitochondrial *b* cytochromes [4]. Here we are concerned with the effects of the bc_1 inhibitors on the optical absorption spectra of *b* cytochromes.

Earlier studies on this point were confined to the α-band of the reduced cytochromes *b*. It has been generally agreed that antimycin shifts the α-peak of ferrocytochrome *b*-562 to the red by less than 1 nm [5]. As regards other inhibitors, the published data are much less consistent or sometimes controversial (e.g., cf. [6–9]), which can be due in part to different objects or preparations used in different laboratories.

Here we describe optical absorption difference spectra induced by antimycin, NoHOQnO, funiculosin, myxothiazol and mucidin in the α-, β- and γ-bands of the reduced and oxidized *b*-cytochromes in the isolated cytochrome bc_1 complex from bovine heart. The spectral effects of the antibiotics were used subsequently to study competition between them for binding with complex bc_1.

2. METHODS

Cytochrome bc_1 complex was isolated from beef heart mitochondria according to Rieske [10]. Antimycin and NoHOQnO were from Serva.

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Abbreviations: NoHOQnO, 2-nonyl-4-hydroxyquinoline-*N*-oxide; SMP, submitochondrial particles

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Mucidin, myxothiazol and funiculosin were kind gifts from Dr V. Musilek (Institute of Microbiology Acad. Sci. CSSR, Prague), Dr W. Trowitzch (Gesellschaft für Biotechnologische Forschung, Braunschweig) and Dr P. Bollinger (Sandoz, Basel). Other chemicals were commercial products of the purest grade available from Sigma, Serva and Merck. Optical measurements were made in an Aminco DW2a spectrophotometer at a slit width of 2 nm in rectangular 10 mm cells thermostatted at 25°C. The basic medium contained 0.3 M sucrose, 30 mM Hepes–NaOH (pH 7.5), 0.02% Na-cholate, and 1 mM EDTA. The same medium, but without cholate, was used in the experiments with SMP. In the experiments with the oxidized complex bc_1, 0.3 mM ferricyanide was added; reduction of the cytochromes was achieved with small amounts of solid dithionite. Typically, experiments were performed as follows: the oxidized or reduced suspension of complex bc_1 (0.5 mg protein/ml) was divided between the sample and reference cells. After correction of the baseline, an inhibitor was added to the sample and an equal volume of the solvent (ethanol or dimethylsulfoxide) to the reference and after 2–3 min incubation the difference spectrum was recorded. Subsequent addition of the inhibitor to the reference always cancelled the absorption difference.

3. RESULTS AND DISCUSSION

Typical difference spectra observed upon addition of various inhibitors to the reduced and oxidized cytochrome bc_1 complex are shown in figs 1 and 2. Very similar difference spectra have also been obtained in experiments with submitochondrial particles [11,12]. In the reduced complex bc_1, all the antibiotics tested bring about well defined spectral changes in the α-, β- and γ-absorption bands (fig.1). In the oxidized preparation, the effects were generally of a smaller size so that in the visible region only antimycin induced a discernible difference spectrum (spectra *a*, dotted line, in fig.1A); however, in the Soret region the spectral changes were quite significant (fig.2). Comprehensive discussion of the spectral effects induced by various inhibitors will be given presently in conjunction with magnetic circular dichroism studies carried out in collaboration with Dr A.M. Arutjunjan's laboratory ([13], in preparation). Some comments on the results are given below.

First of all, it is likely that all the difference spectra induced by the inhibitors are associated predominantly with cytochromes *b* since in all cases they are located around the maxima of the corresponding absorption bands of these hemoproteins.

The effects of antimycin and funiculosin are mirror images of each other throughout the entire spectral range. As it is generally agreed that antimycin shifts to the red ferrocytochrome *b*-562 α-absorption band [5], it is reasonable to assume that funiculosin brings about a blue shift of *b*-562 spectrum of approximately the same magnitude. Also the derivative-shaped effect of NoHOQnO, first observed in [14], centred at 562 nm in the α- and at 430 nm in the Soret band, can be ascribed provisionally to a red shift of ferrocytochrome *b*-562. Indeed, preliminary simulations of the difference spectra [11] show that the effects observed in the α-band fit well to the ferrocytochrome *b*-562 α-peak (Lorentzian shape assumed [11,15]) being shifted by 0.4–0.5 nm and 0.06 nm to the red by antimycin and NoHOQnO, respectively, and by 0.4–0.5 nm to the blue by funiculosin.

Two comments are necessary due to the results obtained with myxothiazol and mucidin. First, we have found that with fresh preparations of complex bc_1 and at low detergent concentrations the spectral changes induced by myxothiazol are identical with those observed in submitochondrial particles [6,11,15] and different from those reported by Von Jagow and Engel for complex bc_1 [7]. However, a more or less symmetrical difference spectrum, similar to the one reported in [7] and closely resembling the effect of mucidin here (fig.1A,d) and in [14], could be observed occasionally with myxothiazol when aged preparations of complex bc_1 were used in experiments or the medium contained high concentrations of detergent. Second, the spectral effect of myxothiazol in [7] (which is approximately equivalent to the effect of mucidin as mentioned above) was postulated without arguments to originate in a red-shift of ferrocytochrome *b*-566 α-band [7]. We would note, that the line shapes of the responses elicited by either myxothiazol or mucidin can hardly support this claim. Moreover, spectral simulations, MCD measurements and experiments at

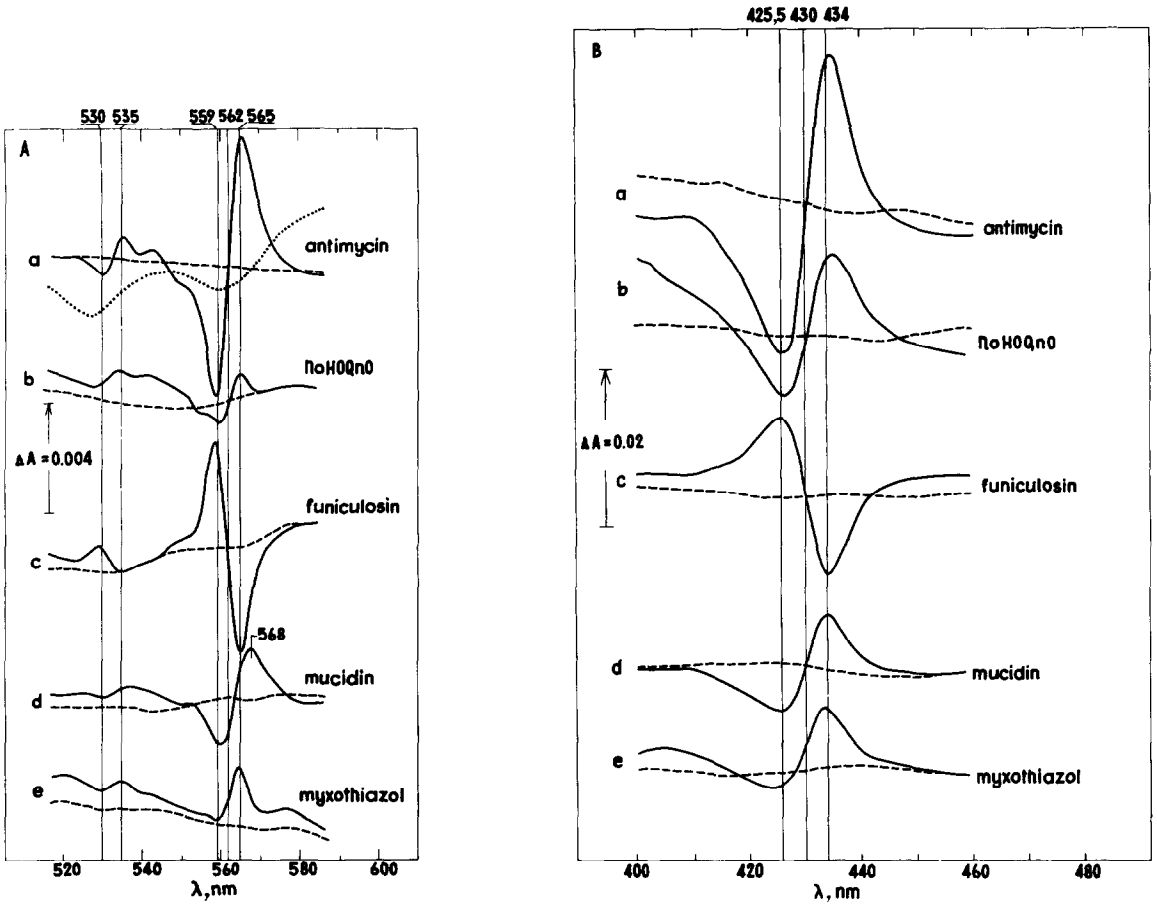

Fig.1. Effects of bc_1-site inhibitors on the optical absorption spectrum of the reduced cytochrome bc_1 complex in the visible (A) and Soret (B) region. The sample and reference cells contained 0.5 mg protein/ml of complex bc_1 in the basic medium to which 1–2 mg solid dithionite was added. After correction of a baseline (– – –), an inhibitor was added to the sample and an equal volume of the solvent to the reference. Inhibitor concentrations: antimycin, 3 μM; NoHOQnO, 20 μM; funiculosin, 8 μM; mucidin, 16 μM; myxothiazol, 3.6 μM. The mutual position of the baselines and corresponding difference spectra on the vertical axis have been chosen arbitrarily. The effect of antimycin on the α,β-band of the oxidized complex bc_1 is also included in the figure (···); in this case conditions are as in fig.2 except that concentration of complex bc_1 is 3.1 mg protein/ml and instrument sensitivity is 3-fold higher than indicated for the other spectra.

controlled redox states of cytochromes *b*-566 and *b*-562 (the results will be described in a full-length paper in collaboration with A.M. Arutjunjan's laboratory) all show consistently that the effects of mucidin and myxothiazol are dominated by the response of cytochrome *b*-562, with a minor contribution from *b*-566.

The spectral changes induced by the antibiotics were further used here to study competition between the inhibitors for binding with the bc_1-site. The results obtained are given in table 1 and can be summarized as follows:

(i) Antimycin, funiculosin and NoHOQnO do not compete with myxothiazol and mucidin (see also [6,7]).

(ii) There is mutual competition between antimycin, funiculosin and NoHOQnO for the binding site; displacement of one inhibitor by the other could be observed in accordance with the binding

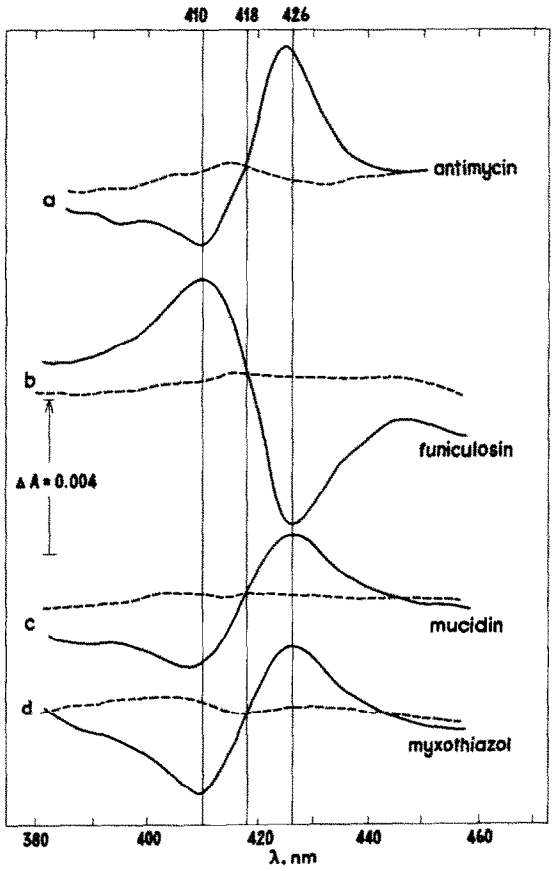

Fig.2. Changes in the Soret band of the oxidized complex bc_1 induced by antimycin, funiculosin, mucidin and myxothiazol. Conditions as in fig.1, but the incubation medium contained 300 μM ferricyanide instead of dithionite. The effect of NoHOQnO is missing in the figure because of the artifactual absorbance changes brought about under these conditions by the inhibitor [12].

strength series antimycin > funiculosin > NoHOQnO which agrees with the inhibitory efficiency of the antibiotics [5,11,17,18].

(iii) Myxothiazol and mucidin compete with each other; displacement of mucidin by myxothiazol is probable since the latter binds the respiratory chain much more tightly [6,11,19] but it is difficult to elicit it spectrophotometrically because the spectral changes induced by the two antibiotics do not differ enough from each other.

In conclusion, the results given here allow us to divide the inhibitors studied into 2 groups. Antimycin, NoHOQnO and funiculosin compete for the same specific binding site and bring about spectral changes which can be attributed to cytochrome *b*-562. Myxothiazol and mucidin compete between themselves but not with the antimycin group and

Table 1

Interdependence of the spectral effects of bc_1-site inhibitors

Inhibitor present initially	Induction of the spectral effect by the inhibitor added				
	Antimycin	Funiculosin	NoHOQnO	Myxothiazol	Mucidin
Antimycin	no	no	no	yes	yes
Funiculosin	yes[a]	no	no	yes	yes
NoHOQnO	yes	yes[a]	no	yes	yes
Myxothiazol	yes	yes	yes	no	no[b]
Mucidin	yes	yes	yes	no[b]	no

[a] A very slow displacement of the inhibitor present is initially observed

[b] The data of the experiments in the Soret band where the spectral changes induced by mucidin and myxothiazol are virtually identical so that displacement of one inhibitor by the other is not spectrophotometrically visible

Conditions essentially as in fig.1 but the sample and reference cells contained initially one of the inhibitors given in the left column. The baseline adjusted, a second inhibitor was added to the sample (and an equal volume of the solvent to the reference) and spectral response was registered

induce a more complex type of spectral change which probably involves contributions from both *b*-566 and *b*-562. This is in agreement with the Q-cycle scheme [20] according to which there can be 2 different sites of action for the inhibitors of electron transfer between cytochromes *b* and c_1. Antimycin NoHOQnO and funiculosin are likely to inhibit cytochrome *b*-562 oxidation in centre *i* of the Q-cycle [3,11,20–22]. Hence it may seem natural that they affect specifically the optical as well as redox [11,23,24] properties of cytochrome *b*-562. As to myxothiazol and mucidin, these are probably inhibitors of QH_2 oxidation by FeS Rieske in centre *o* of the Q-cycle [21,25,26] (rather than of ubisemiquinone oxidation by *b*-566 as suggested in [7]); nevertheless, these antibiotics perturb the spectra and redox potentials [11,24] of both cytochromes *b*. We would remark that the spectral or redox effects of various antibiotics on cytochromes *b* do not necessarily have to be direct and could well be mediated by the structural rearrangements in complex bc_1 induced by these ligands.

Finally, whatever the mechanism, intensive spectral changes produced by the inhibitors can be a simple and useful tool in the studies of their binding with complex bc_1 in different oxidation states.

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